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COMPLETE SPECIFICATION

TREATMENT OF DAIRY PRODUCTS

~~I~~/WE, UNION COOPERATIVE AGRICOLE LAITIERE DE LA MANCHE, a French corporation, of Sottevast 50820 BRIX (Manche) France

hereby declare the invention, for which ~~I~~/we pray that a patent may be granted to ~~me~~/us, and the method by which it is to be performed, to be particularly described in and by the following statement:-

Generally speaking, the invention relates to the treatment of dairy or casein factory whey, Its object is more particularly a process permitting the extraction of glycoproteins and/or sialic acid from such a whey.

It is known that dairy whey is a yellowish liquid which, after its fat content has been removed by centrifugation,consists mainly of lactose, proteins and minera salts.

Treatments have already been proposed for dairy whey in order to render it non-polluting and to recover the proteins it contains. Large amounts of whey are produced by dairies and cheese factories, dairy whey being produced from milk after enzyme treatment and notably after traditional renneting. It has thus been suggested that the proteins should be separated from the whey by ultrafiltration.

However, up to now, ultrafiltration has not been used for separating and obtaining certain specific proteins or other compounds which are of great interest in themselves.

This is notably the case of sialic acid, also called N-acetyl neuraminic acid (see, for example, MERCK Index, 7th Edition,p.715). It is known that sialic acid is present in animal carbohydrate-protein complexes. In actual fact,this compound is at present prepared either from natural raw materials such as the sub-maxillary glands of bovines, or eggs, or by synthesis.

As a bibliographical reference in this connection may be mentioned the articles by M.W. WHITEHOUSE and F. ZILLIKEN"Isolation and Determination of Neuraminic(sialic)

acids",p.199 to 220 in "Methods of Biochemical analysis", Volume VII (1960) Interscience, John Wiley Sons.

These known processes for the preparation of sialic acid are extremely costly,which is passed on to the market price of the product.

As bibliographical references for certain applications of sialic acid,and particularly of NANA,the following articles may be mentioned:

-"Coagulation of milk with rennet: Scientific and technical aspects" GARNIER, MOCQUOT, RIBADEAU-DUMAS,MAUBOIS-Ann' de Nutrition Alimentaire,1968,22, B 495 - B 552.

-Svennerholm L. Acta Soc. Med. Upsaliensis, 61, 75 (1956) Arkiv. Kemi., 10,577 (1956)

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-Werner I. and L. ODIN, Acto Soc. Med.Upsaliensis 57,230 (1952)

-Aminoff, D.(1961) Biochem J. 8L,384

"The sensitivity of the Neuraminosidic Linkage in Muco-substances towards Acid and towards Neuraminidase Gibbons". Biochemistry Journal (1963)89,380.

-"Structure studies on the Myxovirus Hemagglutination Inhibitor of Human Erythrocytes".
Ralph H. Kathan and Richard J. Winzler.
Journal of Biological Chemistry (1963) vol.238 N°1 p.21

-"Studies on the Neuraminidase of Influenza virus II additional properties of the enzymes from the Asian and PR 8 strains".

Max. E. Rafelson, J.R. Michael Schneir and Wannie W. Wilson, J.R. Archives of Biochemistry and Biophysics 103 (1963) 424-430.

Other possible uses of sialic acid are given in the literature relating to this compound.

In another connection, it is advantageous to be able to obtain glycoproteins owing to the possibility of their application in cosmetic compositions.

An object of the present invention is a process for the treatment of dairy whey which makes it possible to obtain sialic acid very cheaply, and more specifically N-acetyl neuraminic acid (abbreviated to NANA) jointly with glycopeptides and a protein fraction consisting of glycoproteins.

The invention therefore relates to a process for the extraction of glycoproteins, glycopeptides and sialic acid from dairy or casein factory whey, with ultrafiltration of said whey, characterized by the steps of:

(a) ultrafiltration of dairy whey through membranes having a cut-off of 10,000 to 50,000 in molecular weight, providing an ultrafiltrate containing essentially lactose, mineral salts and glycopeptides, and a retentate comprising proteins and containing, among other things, sialic acid;

(b) thermal flocculation of the proteins of said retentate providing a first protein precipitate and a first supernatant, which is separated and recovered;

(c) reaction of the first supernatant with phosphotungstic acid under conditions capable of forming a second supernatant and a second protein precipitate, which is separated and recovered;

(d) hydrolysis of the second precipitate, providing a third precipitate and a third supernatant, which is separated and recovered;

(e) extraction of the sialic acid contained in said third supernatant, by known means, involving essentially steps of neutralization, passing the last supernatant over cationic resin, fixing of sialic acid by passing it over an anionic resin, elution of the acid so fixed and recovery of an extremely pure sialic acid by freeze-drying the sialic acid solution produced.

As raw material for the process of the invention, dairy or casein factory whey is used which may be obtained from all ruminants' milk (cows, goats, ewes, buffalos and the like), notably obtained after enzymatic conversion, notably after cows or ewes milk has been renneted. It is also possible to use dehydrated dairy or casein dairy or casein factory whey, as is usual during its conservation, the dry product having water added to it before its use in the process for reconstituting a liquid whey.

In step "a", the dairy or casein factory whey is subjected to ultrafiltration on membranes having an average cut-off expressed in molecular weight of between 10,000 and 50,000. With this in view, all types of membranes now available on the market or which can fulfil the above-mentioned conditions may be used. Organic or inorganic membranes may be used, or even ceramic or metallic screens.

Such membranes allow the lactose molecules, mineral

salts and glycopeptides to pass through and retain the proteins. For industrial requirements it is, in fact, advantageous to recover the ultrafiltrate in a further treatment.

The practical conditions of ultrafiltration are conventional and can be adapted by a man skilled in the art to each specific case. For example, the whey may be flowed at sufficient speed at right angles to the membrane and under a pressure in the range of 3 bars, the product contacted with the membrane being recycled until the protein content of the retentate is optimal, which enables the progress of ultrafiltration to be known . As an example, the membranes "IRIS 3042" made by the French firm RHONE POULENC, which have a cutt-off of about 15,000, are used in the ultrafiltration modules also sold by the aforesaid firm.

It is also possible to use the "DIAFLO" membranes sold by the firm AMICON (USA), such as the membranes DIAFLO XN 50 (cut-off = 50,000), DIAFLO PM 30 (cut-off = 30,000) and DIAFLO PM 10 or UM (cut-off = 10,000).The man skilled in the art will find in the technical handbooks issued by the makers of these membranes all the necessary information on their structure and method of use.

During further treatment,the ultrafiltrate is advantageously subjected to another ultrafiltration with membranes having a cutt-off in the range of about between 1000 and 5000, expressed in molecular weight,such as a cutt-off of

4000 for instance. It is, for example, possible to use the membrane sold under the name DIAFLO UM 2 (cut-off = 1000).

This further ultrafiltration provides a retentate containing glycopeptides which are a valuable product as, for example, an additional nutrient for human and animal feeding. The retentate only needs to be concentrated to provide a syrup of glycopeptides usable in practice, and the ultrafiltrate obtained from said further ultrafiltration essentially comprises lactose which can also be recovered after concentration. It should, furthermore be noted that, instead of being recovered in the form of glycopeptides, the retentate can be subjected to a treatment for the extraction of sialic acid under conditions similar to those which will now be described.

The retentate obtained in step "a" contains sialic acid and, more specifically, NANA. Before subjecting the retentate to the following step "b", it may be advantageous to adjust its protein concentration, which generally involves diluting the retentate with water until a dry matter content of approximately 10% is obtained.

During step "b", selective denaturation of soluble proteins is carried out by thermal flocculation. The albumin and globulines are thus precipitated and the peptone proteases which are glycoproteins are retained in the supernatant. The conditions of this treatment involve heating to a temperature and for a time sufficient to obtain precipitation of the proteins other than the sialoglycoproteins.

Suitable thermal treatment conditions involve, for example, a temperature of 95°C and a duration of 30 minutes. If lower temperature are used, the duration of treatment should be correspondingly lengthened. There is thus obtained a precipitate of proteins which can be recovered and a supernatant containing NANA which is subjected to the following steps of the process. To facilitate separation, the product is cooled, for example to a temperature of 4°C, which is the normal temperature in a refrigerator. The supernatant and protein precipitate are then separated by any suitable means and preferably by centrifugation. The supernatant obtained in step "b" is then contacted with an agent capable of precipitating all the proteins which it still contains. Phosphotungstic acid is used for this purpose; trichloroacetic acid, the reagent known for the precipitation of proteins, is not suited to the requirements of the present process as it only precipitates a portion of the proteins in the supernatant. The conditions of phosphotungstic treatment are not crucial, but it is preferable to operate at ambient temperature. The operation may be effected in an acid medium for example, in the presence of sulphuric acid. The concentration of phosphotungstic acid can also be determined by a preliminary trial. Notably, amounts of approximately 5 g of phosphotungstic acid per liter of supernatant are used, it being understood that larger amounts can be used but do not provide any advantages and cost more. Phosphotungstic treatment is effected for a time

sufficiently long to obtain precipitation of the proteins in the supernatant. Under the conditions previously described, for example, this period of time lasts for a few minutes, for example, 5 minutes at 25°C.

Following treatment with phosphotungstic acid, the protein precipitate is separated from the supernatant by any suitable means, by centrifugation for example. The precipitate is thus recovered and the supernatant expelled. During step "d" the precipitate separated out in step "c" is hydrolyzed. Hydrolysis can be effected by the acid, enzymatic or basic way , but acid hydrolysis is preferably used. It is preferable to use sulphuric acid, or any other acid capable of forming easily precipitable salts after the subsequent neutralization step. Hydrochloric acid is less suitable for this as it provides chloride ions which are difficult to remove subsequently. Acid hydrolysis is advantageously effected at high temperatures, but which should not be higher than about 98°C. The step is, for example, carried out at about 90°C. The acid is used at a moderate concentration, notably at less than 0.5N and, for example, of 0.1 N. Hydrolysis is continued for a time sufficient for said hydrolysis to be complete; this is generally about one hour.

After cooling, the product subjected to hydrolysis provides a precipitate and a supernatant, which are separated by any conventional means, notably by centrifugation. Said precipitate is eliminated, whereas the supernatant is recovere

The further treatment of said supernatant, corresponding to step "e" of the process, comprises a certain number of operations enabling the NANA to be extracted. At this stage of the treatment of dairy whey, the invention makes use of the known technique for obtaining sialic acid. The supernatant is first neutralised with a view to precipitating, in the form of insoluble salts, the free SO_4^{--} ions still present in the supernatant. This step is advantageously effected by the addition of ~~baryum~~ barium hydroxide in excess for precipitating the sulphate ions if hydrolysis was effected with sulphuric acid.

An excess of ~~baryum~~ barium ions is used until there is obtained a pH approximating neutral. The precipitated salts thus formed, such as ~~baryum~~ barium sulphate, are then removed and the supernatant is retained. This is optionally concentrated before being flowed through the resin columns. Cationic resin is used for the first flow through in order to demineralize the supernatant. For example, resins available on the market under the name "DOWEX" are used, such as type AG 50 WX 8 H +. After passing over the cationic resin, the product is caused to flow through an anionic column in order to fix the NANA. Suitably, the resin available on the market under the name of DOWEX type AG 1 X8 formate is used. The NANA is then obtained from the said anionic resin after washing the column with distilled water by elution notably with formic acid such as 0.3M formic acid if an anionic resin in the formate form is used.

Finally, a solution is obtained which, by freeze drying, provides an extremely pure NANA powder. The operation constituting treatment "e" can undergo variations. For example, after neutralization, separation of ~~baryum~~ barium sulphate and clarification of the supernatant, the last can be dried. The powder obtained is then subjected to solvent extraction, that is to say, it is mixed with a solvent or mixture of solvents in which NANA is soluble, such as ethanol or an ethanol-water mixture. The NANA extracted is then isolated by elimination of the solvent.

The drawing illustrates the succession of steps of the process of the invention in a practical form of embodiment. Insofar as the raw materials are concerned, the dairy whey B is obtained either by adding rennet to milk A, or from powdered whey B' rehydrated for this purpose. The succession of steps can be clearly followed on the drawing. It should be noted that after ultrafiltration 1, the ultrafiltrat obtained is subjected to a further ultrafiltration 1-2, the retentate of which contains glycopeptides, which are one of the products obtained by the process of the invention.

After step 4 (separation) a supernatant containi glycoproteins is obtained, these are another valuable product.

In step "6", the abbreviation PTA designates phosphotungstic acid. The last step of the process (freeze-drying 21) provides NANA, which is another product sought for, and obtained by the invention.

The invention will now be illustrated by examples of embodiments of the process.

EXAMPLE 1-

1000 liters of cows milk was renneted in the traditional manner and 900 liters of dairy whey were obtained. These 900 liters of whey were flowed through an ultrafiltration module put on the market by the firm RHONE POULENC and provided with an IRIS 3042 membrane having a cut-off of 15,000. The whey was introduced into the module at a pressure of 3 bars and a température of about ambient temperature.

870 liters of ultrafiltrate containing glycopeptides was thus obtained. Said ultrafiltrate was placed in an ultrafiltration module provided with a membrane having a cut-off of 1000. The retentate obtained from this other ultrafiltration was concentrated, which enabled 3700 grammes of a syrup to be obtained having a dry matter content of 30% consisting essentially of glycopeptides. The retentate obtained from the first ultrafiltration is a concentrate of proteins with a 20% dry matter content containing about 100 grammes of NANA. The retentate was diluted until a level of 10% dry matter was obtained, the proteins then being flocculated by heating at 95°C for 30 seconds. The product was then cooled to 4°C, then centrifuged until there was obtained a supernatant having a volume equal to 60% of the initial volume, which represents about 50 grammes of NANA. This supernatant was treated by the addition of phosphotungstic

acid at a rate of 5 grammes per liter and the reaction was continued with stirring for 5 minutes at 25°C. The precipitate obtained was separated and the supernatant eliminated. The precipitate contained 22 grammes of NANA.

This precipitate has been subjected to acid hydrolysis with 0.1 N sulphuric acid for 60 minutes at a temperature of 90°C . To facilitate separation of the precipitate the product was cooled to 4°C and centrifugation was used to separate the precipitate from the supernatant. The precipitate was expelled and the treatment was continued with the supernatant which contained about 20 grammes of NANA. This supernatant was then neutralized by the addition of a saturated aqueous solution of ~~baryum~~ barium hydroxide until a neutral pH was obtained, which resulted in the precipitation of the excess sulphate ions in the form of ~~baryum~~ barium sulphate. The solution was then clarified and the ~~baryum~~ barium sulphate removed. The supernatant which was reserved contained 19 grammes of NANA. Said supernatant was concentrated, for example by reducing its volume by 4 to 6-fold, using a rotating evaporator with vacuum, at a temperature of 45°C, the pressure being from 20 to 30 mm Hg. In order to demineralize the concentrated solution so obtained, it was flowed through a cationic resin column available on the market under the name of DOWEX, type AG 50 WX 8H+. At the output of said column and in order to fix the NANA, the solution is flowed through an anionic resin column of the type DOWEX AG 1 X 8 formate. Double distilled water was then used to wash the column containing the anionic resin and the NANA was

eliminated with 0.3 N formic acid. 70% of the NANA fixed on the anionic resin was thus recovered. Freeze drying of the formic solution provided 13 grammes of extremely pure NANA powder.

The economic value of the process is proved by the amount of NANA produced.

EXAMPLE 2-

The operation was conducted under conditions identical to those used in example 1, but starting with 1000 liters of ewes milk, the results were substantially equivalent.

EXAMPLE 3-

The operation was conducted as in example 1 but starting with a liquid whey obtained by the regeneration of powdered whey, 50 kg of whey powder was used, diluted to provide 900 liters of liquid whey.

WHAT WE CLAIM IS:

1. A process for the extraction of glycoproteins, glycopeptides and sialic acid from dairy or casein factory whey, with ultrafiltration of said whey, characterized by the steps of:

(a) ultrafiltration of dairy whey through membranes having a cut-off of 10,000 to 50,000 in molecular weight, providing an ultrafiltrate containing essentially lactose, mineral salts and glycopeptides, and a retentate comprising proteins and containing, among other things, sialic acid;

(b) thermal flocculation of the proteins of said retentate providing a first protein precipitate and a first supernatant, which is separated and recovered;

(c) reaction of the first supernatant with phosphotungstic acid under conditions capable of forming a second supernatant and a second protein precipitate, which is separated and recovered;

(d) hydrolysis of the second precipitate, providing a third precipitate and a third supernatant, which is separated and recovered;

(e) extraction of the sialic acid contained in said third supernatant, by known means, involving essentially steps of neutralization, passing the last supernatant over cationic resin, fixing of sialic acid by passing it over an anionic resin, elution of the acid so fixed and recovery of an extremely pure sialic acid by freeze-drying the sialic acid solution produced.

2. A process according to claim 1, characterized in that, as raw material, a whey is used obtained by traditional renneting of ruminants milk or a reconstituted whey obtained by the addition of water to a dehydrated dairy whey.

3. A process according to claim 1 or 2, characterized in that during the ultrafiltration of step (a) the whey is flowed over the membrane under a pressure of 3 bars, the product contacted with the membrane being recycled until an optimal protein content of the retentate is obtained.

4. A process according to any of claims 1 to 3, characterized in that the ultrafiltrate obtained from step (a) is subjected to another ultrafiltration with membranes having a cut-off in the range of 1000 to 5000, expressed in molecular weight, thus providing a retentate containing all the glycopeptides and an ultrafiltrate comprising essentially lactose and mineral salts.

5. A process according to claim 4, characterized in that the retentate is recovered to form a concentrate of glycopeptides or subjected to a treatment for the extraction of sialic acid under the same conditions as those of the retentate obtained in step (a).

6. A process according to any of claims 1 to 5, characterized in that, before step (b), the retentate obtained in step (a) is diluted by the addition of water to adjust its protein concentration, notably until a dry matter content of approximately 10% is obtained.

7. A process according to any of claims 1 to 6, characterized in that, during step (b), selective denaturation of the soluble proteins in the retentate, optionally diluted, is effected, the conditions of said flocculation involving heating to a temperature and for a length of time sufficient to obtain the precipitation of albumins and globulins without denaturing the peptone proteases (sialoglycoproteins).

8. A process according to claim 7, characterized in that the thermal flocculation of the retentate is effected at about 95°C, for about 30 minutes.

9. A process according to any of claims 1 to 8, characterized in that, to facilitate separation of the flocculate obtained after the thermal flocculation, the product is cooled, notably to 4°C.

10. A process according to any of claims 1 to 9, characterised in that the reaction with phosphotungstic acid is effected in an acid medium.

11. A process according to any of claims 1 to 10, characterized in that, in step (c), an amount of approximately 5g of phosphotungstic acid per liter of the supernatant is used.

12. A process according to any of claims 1 to 11, characterized in that, during step (d) the precipitate separated in step (c) is hydrolyzed, the hydrolysis being effected by acid, enzyme or base.

13. A process according to claim 12, characterized in that an acid hydrolysis step is effected with acid, notably with sulphuric acid, at a concentration lower than 0.5N.

14. A process according to either claim 12 or 13, characterized in that hydrolysis is effected at a temperature not higher than 98°C.

15. A process according to any of claims 1 to 14, characterized in that, during extraction (e), the supernatant is neutralized in order to precipitate the free acid ions in the form of salts, preferably by the addition of excess barium hydroxide, to precipitate the sulphate ions if hydrolysis was effected with sulphuric acid.

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16. A process according to any of claims 1 to 15, characterized in that, during extraction (e), the neutralized, clarified and optionally concentrated supernatant is flowed over a cationic resin then over anionic resin after which the anionic column is washed and eluted to recover the sialic acid fixed on the said resin, elution being with the acid having the same anion as the anion used in the anionic resin.

17. A process for the extraction of glycoproteins, glycopeptides and sialic acid from dairy or casein factory whey, with ultrafiltration of said whey, characterized by the steps of:

(a) ultrafiltration of dairy whey through membranes having a cut-off of 10,000 to 50,000 in molecular weight, providing an ultrafiltrate containing essentially lactose, mineral salts and glycopeptides, and a retentate comprising proteins and containing, among other things, sialic acid;

(b) thermal flocculation of the proteins of said retentate providing a first protein precipitate and a first supernatant, which is separated and recovered;

(c) reaction of the first supernatant with phosphotungstic acid under conditions capable of forming a second supernatant and a second protein precipitate, which is separated and recovered;

(d) hydrolysis of the second precipitate, providing a third precipitate and a third supernatant which is separated and recovered;

(e) extraction of the sialic acid contained in said third supernatant, by known means, involving essentially the steps of neutralization, and clarification of the supernatant which is then dried and the powder obtained subjected to solvent extraction, by putting the said powder into intimate contact with a solvent or mixture of solvents in which the sialic acid is soluble, separating the so-formed sialic acid solution and eliminating the solvent or solvent mixture to give sialic acid.

18. The sialic acid obtained by the process according to any of claims 1 to 16, notably freeze dried and of extreme purity.

19. The sialic acid obtained by the process according to claim 17, notably freeze dried and of substantial purity.

20. The protein fractions, notably the glycoproteins and glycopeptides, obtained by the process according to any of claims 1 to 5.

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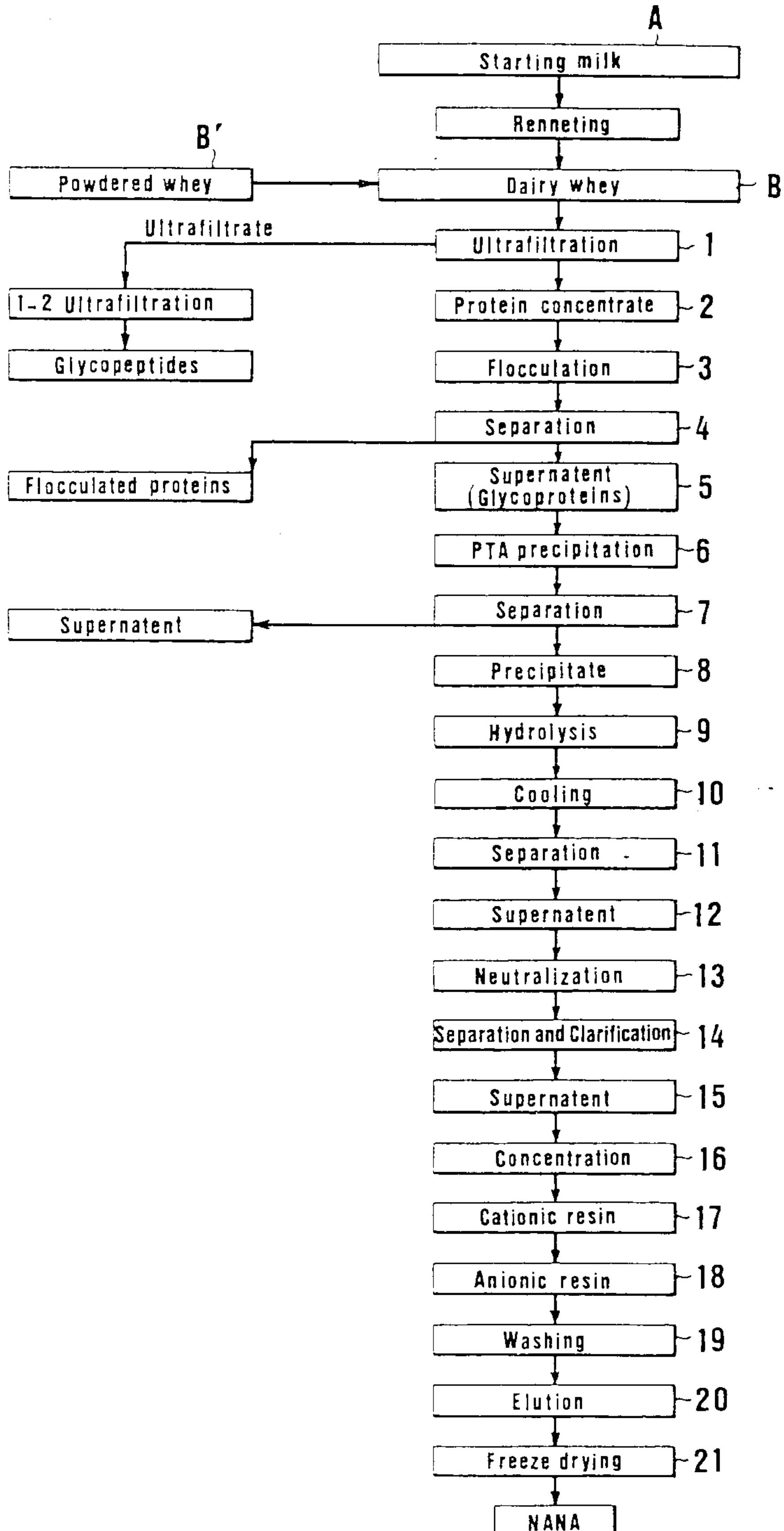

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